Off balance: Regulatory and effector T cells in the pathogenesis of ANCA associated vasculitis

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CHAPTER 5

Urinary and serum soluble CD25 complements urinary soluble CD163 to detect active renal anti-neutrophil cytoplasmic autoantibody associated vasculitis.

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Abstract

Background: Early detection of renal involvement in anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AAV) is of major clinical importance to allow prompt initiation of treatment and limit renal damage. Urinary soluble (us)CD163 has recently been identified as a potential biomarker for active renal vasculitis. However, a significant number of patients with active renal vasculitis test negative using usCD163. We therefore studied whether sCD25, a T cell activation marker, could improve the detection of renal flares in AAV.

Methods: sCD25 and sCD163 levels in serum and urine were measured by Enzyme-Linked Immuno Sorbent Assay in 72 patients with active renal AAV, 20 with active extra-renal disease, 62 patients in remission and 18 healthy controls. Urinary and blood $CD4^+$T and $CD4^+$T effector memory (TEM) cell counts were measured in 22 patients with renal active vasculitis. ROC curves were generated and recursive partitioning was used to calculate whether usCD25 and serum (ss)CD25 adds utility to usCD163.

Results: usCD25, ssCD25 and usCD163 levels were significantly higher during active renal disease and significantly decreased after induction of remission. A combination of usCD25, usCD163 and ssCD25 outperformed all individual markers (sensitivity: 84.7%, specificity: 95.1%). Patients positive for sCD25 but negative for usCD163 (n=10) had significantly higher C-reactive protein levels and significantly lower serum creatinine and proteinuria levels compared to the usCD163 positive patients. usCD25 correlated positively with urinary $CD4^+$T and $CD4^+$TEM cell numbers whereas ssCD25 correlated negatively with circulating $CD4^+$T and $CD4^+$TEM cells.

Conclusion: Measurement of usCD25 and ssCD25 complements usCD163 in the detection of renal active vasculitis.

Introduction

Anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis (AAV) is a group of systemic autoimmune diseases characterized by pauci-immune necrotizing inflammation of small- to medium-sized blood vessels with a predilection for lungs and kidneys. AAV is the leading cause of rapidly progressive glomerulonephritis, especially in the elderly [1, 2].

Although immunosuppressive treatment induces remission in most patients, more than 30% of patients experience a relapse of disease within the first three years after induction of remission [3, 4]. Eventually, more than 70% of all patients develop renal involvement, which is associated with a decline in renal function and increased morbidity and mortality [5, 6]. Therefore, early detection of renal relapses is important to prevent permanent renal dysfunction in AAV.

A potent new marker for renal active vasculitis, soluble (s)CD163, a macrophage shed scavenger receptor for haemoglobin-haptoglobin, has recently been identified [7]. Increased levels of urine (us)CD163 closely reflect active renal vasculitis and correlate to macrophage infiltration into the kidney [7]. Macrophages are key players in renal damage as they are the most frequent inflammatory cell type present in glomerular crescents [8, 9]. Although high levels of usCD163 reflect renal active vasculitis, between 13 to 27 percent of the patients with renal active vasculitis test negative [7]. Thus, usCD163 levels alone are insufficient to identify renal active vasculitis in all AAV patients.

In addition to macrophages, T cells also play a pivotal role in the pathogenesis of AAV. T cells in peripheral blood of both active AAV patients and patients in remission are persistently activated, and various aberrations in T cell subsets, both numerical and functional, have been identified in AAV patients with active disease [10-16]. Furthermore, increased numbers of $CD4^+$ effector memory T (TEM) cells in the urine have been found to reflect active renal vasculitic disease [17].

Accordingly, T cell activation markers have been proposed as potential disease activity markers, including CD25, the T cell IL-2 alpha receptor (sIL-2Rα) which is significantly higher expressed on $CD4^+$T cells during active disease compared to healthy controls

[16]. Soluble (s)CD25, which is shed from T cells after activation, is increased in serum of AAV patients with active disease and correlated with vasculitis disease activity and decreased upon induction of remission [18-20]. A recent study identified usCD25 as a potential biomarker for active Lupus nephritis, demonstrating increased levels during active disease and in patients in whom induction of disease remission failed [21]. However, in AAV, data on usCD25 levels as a marker for renal disease activity is lacking. Therefore, as both T cell and macrophage activation play a pivotal role in AAV pathogenesis, we studied whether sCD25 could complement usCD163 in the detection of renal active AAV.

Material and methods

Study population

Patients included in the inception cohort were recruited from the University Medical Center Groningen (UMCG). The inception cohort consisted of 24 patients with active renal disease and 5 patients with active non-renal AAV. Of 12 patients paired samples were available that is a samples collected during active disease and a sample collected at disease remission.

For the validation cohort samples of patients from the Rare Kidney Disease (RKD) Biobank, Trinity Healthy Kidney Centre, Dublin, Ireland were used. The validation cohort consisted of 48 patients with active renal AAV, 15 patients with active non-renal AAV and 50 patients in remission. Ten of the 50 patients in remission were also included during active disease. Patient characteristics are listed in Table 1. AAV diagnosis was established according to the Chapel Hill consensus classification criteria [22]. Disease activity was determined using the third version of the Birmingham Vasculitis Activity Score (BVAS) [23]. Active renal vasculitis was defined according to clinical practice as new or increasing hematuria, and/or proteinuria, and/or in serum creatinine. Patients with active disease, but without renal involvement, were defined using BVAS v3 based on the absence of clinical signs of renal involvement.

Table 1: Patient characteristics

	Inception cohort	Validation cohort	HC
Number of patients	41	113	18
Median age (years, range)	57.5 (47.4-67.3)	61.0 (47.9-73.5)	53.8 (42.8-60.9)
Male (n, %)	23 (56.1%)	64 (56.6%)	10 (55.5%)
ANCA specificity			
PR3 (n, %)	27 (65.9%)	66 (58.4%)	-
MPO (n,%)	14 (34.1%)	47 (41.6%)	-
Disease state			
Active – Renal	24 (58.5%)	48 (42.5%)	-
Active – Non-renal	5 (12.2%)	15 (13.3%)	-
Remission	12 (29.3%)	50 (44.2%)	-
Diagnosis/Relapse			
Active – Renal	10/14	42/6	-
Active – Non-renal	1/4	4/11	-
BVAS – Active renal	15 (12-19)	15 (12-19)	-
BVAS – Active non-renal	11 (8-19)	11 (8-16)	-
Serum creatinine (µmol/l)			
Active renal disease	175 (94-319)	266 (120-365)	-
Active non-renal disease	63 (59-128)	88 (68-129)	-
Remission	86 (68-129)	114 (86-165)	-
Proteinuria (g/l)			
Active renal disease	0.8 (0.3-2.2)	0.7 (0.2-2.0)	-
Active non-renal disease	0.4 (0.0-0.6)	0.6 (0.0-0.8)	
Remission	0.2 (0.0-0.4)	0.3 (0.0-0.5)	
Immunosuppressive treatment (n, %)	17 (41.4%)	70 (61.9%)	0 (0%)

Remission was defined as a BVAS of 0, including stable urinary sediment and serum creatinine levels. The study was approved by the local Ethics Committees and informed consent was obtained from all participants in agreement with the declaration of Helsinki.

Sample collection and preparation

For serum, 10 ml of blood was collected and allowed to clot at room temperature for 1 hour, then centrifuged for 10 minutes at 1500 g and stored at either -20 °C (UMCG samples) or -80 °C (RKD samples). For patients recruited in the UMCG, spot urine samples were diluted 1:1 in phosphate-buffered saline (PBS) and centrifuged for 15 minutes at 1200 g, the supernatants were collected and stored at -20 °C until use, whereas the cell pellets of the active AAV patients were used for T cell measurements. Spot urine samples from patients enrolled in the RKD were centrifuged at 2000 g for 10 minutes at 4 °C, and the supernatants were stored at -80 °C until use.

sCD25 and sCD163 detection

Serum and urine sCD25 and sCD163 levels were detected by commercial sandwich enzyme-linked immuno sorbent assay (ELISA) (human sIL-2R DY223, human sCD163 DY1607, R&D systems) according to the manufacturer's instructions with minor modifications. For sCD25, serum samples were diluted 1:2 and urine samples were diluted 1:4 and 1:8 in 1%BSA/PBS.

For sCD163, levels were measured and data were published previously [7]. Data are presented here as well to compare sCD163 with sCD25 and identify patients testing false negative for sCD163. In short, serum samples were diluted 1:200 and 1:400 and urine samples were diluted 1:4 and 1:8 in 1% BSA/PBS. Both the capture antibody and the streptavidin HRP conjugate were diluted in 1% BSA/PBS/0,005% Tween to minimize background signal. Levels of sCD25 and sCD163 in urine were corrected for urinary creatinine levels to correct for urinary dilution.

Phenotyping and quantification of CD4$^+$ T cells in urine and blood samples

CD4$^+$T and CD4$^+$T effector memory (TEM) cell numbers in urine and blood were previously assessed and published [24]. Here, these data were used to correlate T cell numbers and migration to sCD25 levels. In short, immediately after voiding, urine was diluted 1:1 with cold phosphate-buffered saline (PBS) and processed as previously described [17]. Isolated mononuclear cells were resuspended in wash-buffer (1% BSA/PBS) and stained with anti-CD45RO-FITC, anti-CCR7-PE, anti-CD4-PerCP and anti-CD3-APC for 15 minutes at room temperature in the dark. In parallel, blood samples were labeled with the aforementioned monoclonal antibodies. Next, cells were treated with 2 ml diluted FACS lysing solution (BD, Amsterdam, The Netherlands) for 10 minutes and samples were washed twice in wash-buffer and immediately analyzed on FACS-Calibur (BD, Amsterdam, The Netherlands). Data were collected for 10^5 events for each sample and plotted using Win-List software package (Verity Software House Inc., Topsham, USA). Positively and negatively stained populations were calculated by quadrant dot-plot analysis, as determined by the isotype controls.

Absolute numbers of CD4$^+$T-cells were quantified in urine and blood using TrueCOUNT™ Tubes (BD, Amsterdam, The Netherlands). In brief, 20 µl of MultiTEST™ four-color antibodies (CD3-FITC, CD8-PE, CD45-PerCP and CD4-APC) and 50 µl of sample (urine or blood) were added to bead-containing TrueCOUNT™ tubes. The cell suspension was processed and analyzed and absolute counts for CD4$^+$T and TEM cells were calculated as described before [17].

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7 for Windows (GraphPad Software, San Diego California, USA). As sCD25 and sCD163 levels in serum and urine were non-normally distributed, differences between the groups were analyzed using the Kruskal Wallis test with post-hoc Dunn's test. Wilcoxon matched pairs testing was used for paired analysis. Correlations were tested using Spearman's rank correlation. For usCD25, usCD163 and ssCD25 receiver operator curves (ROC) were generated between patients with active renal vasculitis and patients in remission. In

the inception cohort, optimal cut-off values were calculated using Youden index [25]. The optimal cut-off values were then applied to the validation cohort. Recursive partitioning was used to calculate optimal combination of usCD25 and usCD163 and ssCD25 and ssCD163 for detection of renal active vasculitis (R, v3.3.3). Cut-off values derived from the inception cohort were used for this analysis and the optimal combination of markers was applied on the combination of the inception and validation cohort. A p-value of <0.05 was considered significant.

Results

sCD25 levels in urine and serum reflect vasculitis activity

Patients with active renal vasculitis had significantly higher ssCD25 levels compared to patients in remission and healthy controls in the inception cohort and to patients with active non-renal disease in the validation cohort (Fig. 1A). A significant decrease in ssCD25 levels was found in paired samples of patients after achieving remission compared to the levels during active disease (median 937 pg/ml vs 494 pg/ml) (Fig. 1C).

In urine, usCD25 levels were significantly higher in patients with active renal vasculitis compared to healthy controls. Moreover, in the validation cohort usCD25 levels also differed significantly between patients with active renal disease and patients in remission (Fig. 1B). usCD25 levels decreased significantly upon remission (median 210 ng/mmol vs 90 ng/mmol) (Fig. 1D).

Patients with active renal disease who received induction treatment before sampling had significantly lower ssCD25 (median 1369 vs 798 pg/ml) levels and lower usCD25 levels (median 190 vs 59 ng/mmol). Maintenance treatment did not affect ssCD25 or usCD25 levels significantly (Supl. Table 1). ANCA specificity did not influence usCD25 or ssCD25 levels during active disease or in remission (data not shown).

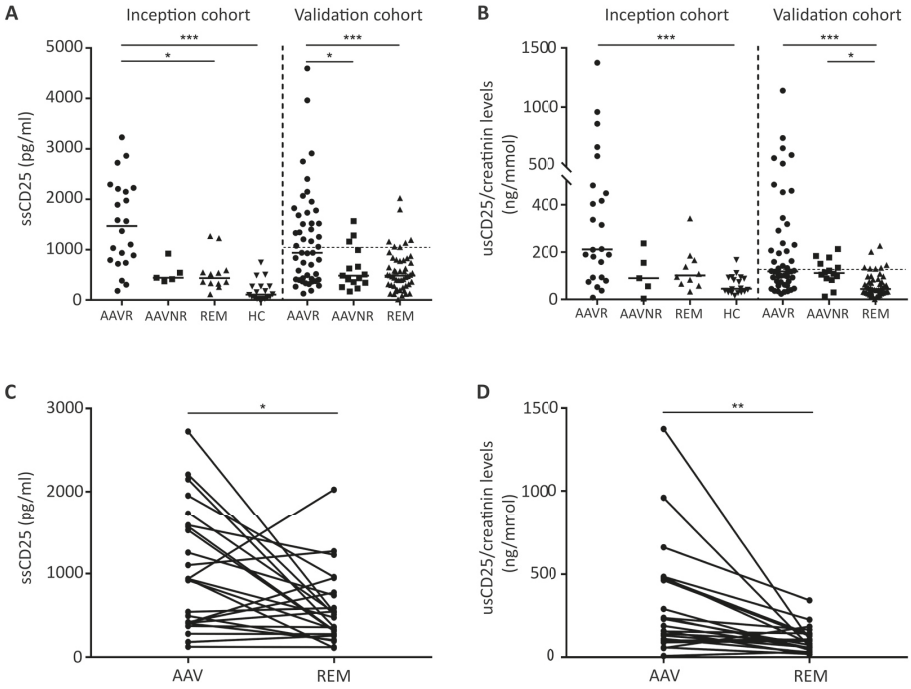

Figure 1: sCD25 levels in patients and healthy controls.
sCD25 levels in serum (A) and in urine (B). Paired sCD25 levels were measured during active disease and remission states in serum (C) and urine (D). AAV R; Patients with renal active disease, AAV NR; Patients with active disease without renal involvement, REM; patients in remission and HC; Healthy controls. (* P<0.05, ** P<0.01, *** P<0.001).

usCD25 correlate with urine CD4⁺T cells

As sCD25 is shed from activated T cells, correlations between ssCD25 and CD4⁺T or CD4⁺TEM cell counts in blood, and correlations between usCD25 and CD4⁺T or CD4⁺TEM cells in urine were tested. A negative trend between ssCD25 levels and circulating CD4⁺T (rho=-0.431, p=0.050) or CD4⁺TEM cells (rho=-0.405, p=0.068) was observed (Fig. 2 A-B). On the other hand, usCD25 levels correlated positively with urinary CD4⁺T (rho=0.481, p=0.048) and CD4⁺TEM (rho=0.430, p=0.046) cell numbers in active renal AAV patients (Fig. 2 C-D).

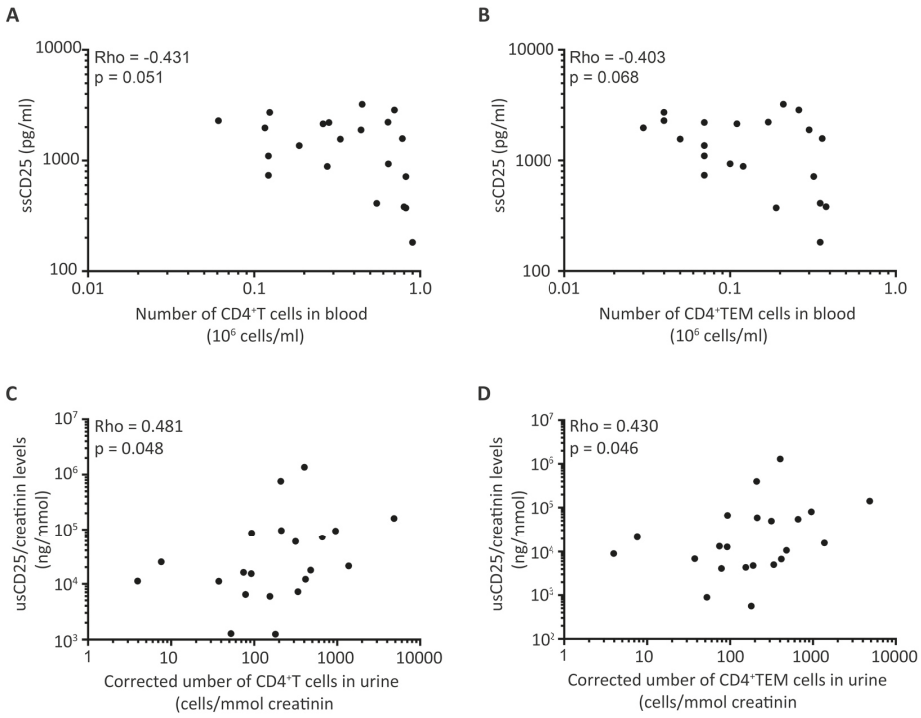

Figure 2: CD25 correlates with T cells and effector memory T cells in serum and urine. Correlations between ssCD25 levels and circulating $CD4^+$T (A) and $CD4^+$TEM cells (B), and correlations between usCD25 levels and urinary $CD4^+$T (C) and $CD4^+$TEM cells (D).

usCD163 levels are highly increased during renal active AAV

Patients with active disease had significantly higher ssCD163 levels compared to healthy controls in the inception cohort (Fig 3A) but levels did not decrease upon induction of remission (median 681 ng/ml vs 495 ng/ml) (Fig. 3C). usCD163 was significantly higher in patients with active renal disease than in patients in remission or healthy controls. In the validation cohort levels of usCD163 also significantly differed between patients with and without active renal vasculitis (Fig 3b) than in all other groups (Fig. 3B), and levels decreased significantly upon induction of remission (median 404 vs 35 ng/mmol) (Fig. 3D). Moreover, the presence of induction therapy at the time of sampling was associated with lower ssCD163 levels (median: 678 vs 255 ng/ml) but did not influence usCD163 levels (median 678 vs 574 ng/mmol) in patients with renal active

disease. Moreover, maintenance treatment did not influence usCD163 or ssCD163 levels significantly (Supl. Table 1).

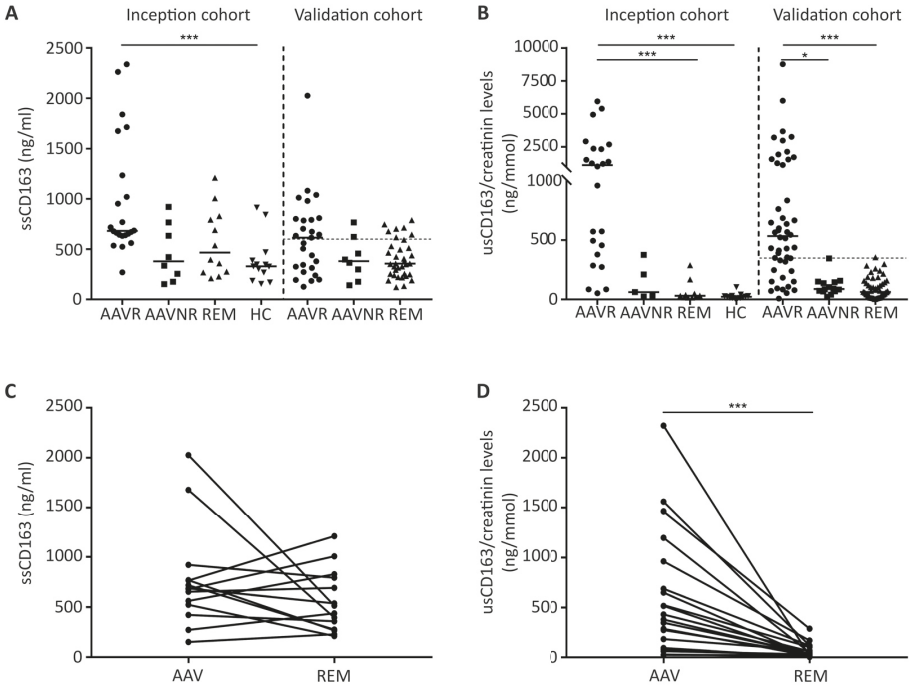

Figure 3: sCD163 levels in patients and healthy controls.
sCD163 levels in serum (A) and in urine (B). Paired sCD163 levels were measured during active disease and remission states in serum (C) and urine (D). AAV R; Patients with renal active disease, AAV NR; Patients with active disease without renal involvement, REM; patients in remission and HC; Healthy controls. (* P<0.05, ** P<0.01, *** P<0.001).

usCD163 reflects renal active AAV

To test whether the soluble markers reflected renal active vasculitis in patients with an established diagnosis of AAV, ROC curves were generated of patients with active renal disease, active non-renal disease and those in remission. Using the inception cohort data, optimal cut-offs for usCD25 (cut-off >125ng/mmol), ssCD25 (cut-off >1050ng/ml), usCD163 (cut-off >350ng/mmol) and ssCD163 (cut-off >630) were calculated (Table 2).

The cut-off values were applied to the validation cohort. In the validation cohort, usCD25 and usCD163 were comparable in the detection of active renal vasculitis

(65.7% vs 70.8%) whereas ssCD25 and ssCD163 were less sensitive (60.0% and 62.5% respectively). In total, 19.6% of the patients that tested positive using usCD25 did not have active renal disease. When usCD163 was used this percentage decreased to 2.5%. ssCD25 and ssCD163 performed less compared to usCD163 (Table 2).

Table 2: Statistical analysis of the different markers

	AUC	Sensitivity	Specificity	PPV	NPV	+LR	-LR	Cut-off
Inception cohort (AAV R = 24, AAV NR/REM = 17)								
usCD25	0.80	70.8	76.5	80.9	65.0	3.0	0.38	>125 ng/mmol
ssCD25	0.82	65.7	80.4	84.2	63.6	3.8	0.40	>1050 pg/ml
usCD163	0.94	75.0	94.1	94.7	72.3	13.0	0.27	>350 ng/mmol
ssCD163	0.70	67.3	56.2	73.1	66.7	1.9	0.35	>630 ng/ml
Validation cohort (AAV R = 48, AAV NR/REM = 65)								
usCD25	0.81	65.7	80.4	67.4	74.6	2.8	0.46	-
ssCD25	0.76	60.0	72.7	61.7	71.2	2.2	0.55	-
usCD163	0.87	70.8	98.5	97.1	82.1	46	0.30	-
ssCD163	0.66	62.5	75.4	65.2	73.1	2.5	0.50	-
Total cohort (AAV R = 72, AAV NR/REM = 82)								
usCD25	0.80	66.7%	78.6%	71.6	72.3	2.9	0.43	-
ssCD25	0.78	61.8%	73.7%	68.2	69.3	2.4	0.50	-
usCD163	0.91	72.2%	97.5%	96.3	80.0	30.0	0.28	-
ssCD163	0.66	66.1%	71.9%	68.1	71.9	2.4	0.44	-
Decision tree	-	84.7	95.1	93.8	87.6	16.0	0.16	-

AUC; area under the curve, PPV; positive predictive value, NPV; negative predictive value, +LR; positive likelihood ration and -LR; negative likelihood ratio

These results indicate that usCD163 acts as the most promising single marker in the detection of renal active disease, however 25.0% of the patients in the inception and 29.2% of the patients with active renal disease in the validation cohort still tested false negative.

The utility of traditional markers for renal active vasculitis (proteinuria, hematuria, serum creatinine and C-reactive protein (CRP)) was also tested. Of these, CRP tested

the most accurate, with a sensitivity of 77.9% and a specificity of 71.6%. However, CRP alone was not able to detect active disease as 55% of the patients without renal disease also tested positive (Supl. Table 2).

usCD25 and ssCD25 complements usCD163 for the detection of renal active AAV

To study whether the sensitivity of detecting renal active vasculitis could be further increased compared to usCD163 alone, we tested various combinations of markers using recursive partitioning. The optimal combination comprised usCD163, usCD25 and ssCD25. In this analysis, patients were tested first for usCD163 and patients with usCD163 levels above the cut-off value were considered to have active renal disease (true positives = 51, false positives = 1) (Fig. 4B). Next, patients that did not reach cut-off for usCD163 were further evaluated using usCD25 and ssCD25. Patients that reached cut-off for both usCD25 and ssCD25 were classified in the tree as having active renal disease (true positives = 10, false positives = 2) (Fig. 4B). Patients who tested negative for all three markers were classified as patients without renal active disease (true negatives = 79, false negatives = 11) (Fig. 4B). This combination led to an increase in sensitivity compared to usCD163 alone (84.7% vs 72.2%, Table 2).

Additional analysis between the three groups revealed that patients that did not reach cut-off for usCD163 but did test positive for usCD25 and ssCD25 (n=10) had significantly higher levels of C-reactive protein (CRP) and significantly lower levels of serum creatinine and proteinuria compared to patients that tested positive for usCD163 (n=51). Moreover, the number of PR3-ANCA positive patients tended to be higher in the us/ssCD25 positive group compared to the usCD163 positive group (p=0.07) (Table 3).

Patients that tested false negative(n=11) had significantly lower serum creatinine compared to the usCD163 positive group and significantly lower CRP compared to the us/ssCD25 positive group (Table 3). Nine out of the 11 (73%) false negative patients received immunosuppressive treatment before sampling, which was higher compared to the usCD163 and us/ssCD25 positive groups (41% and 40% respectively) (Table 3).

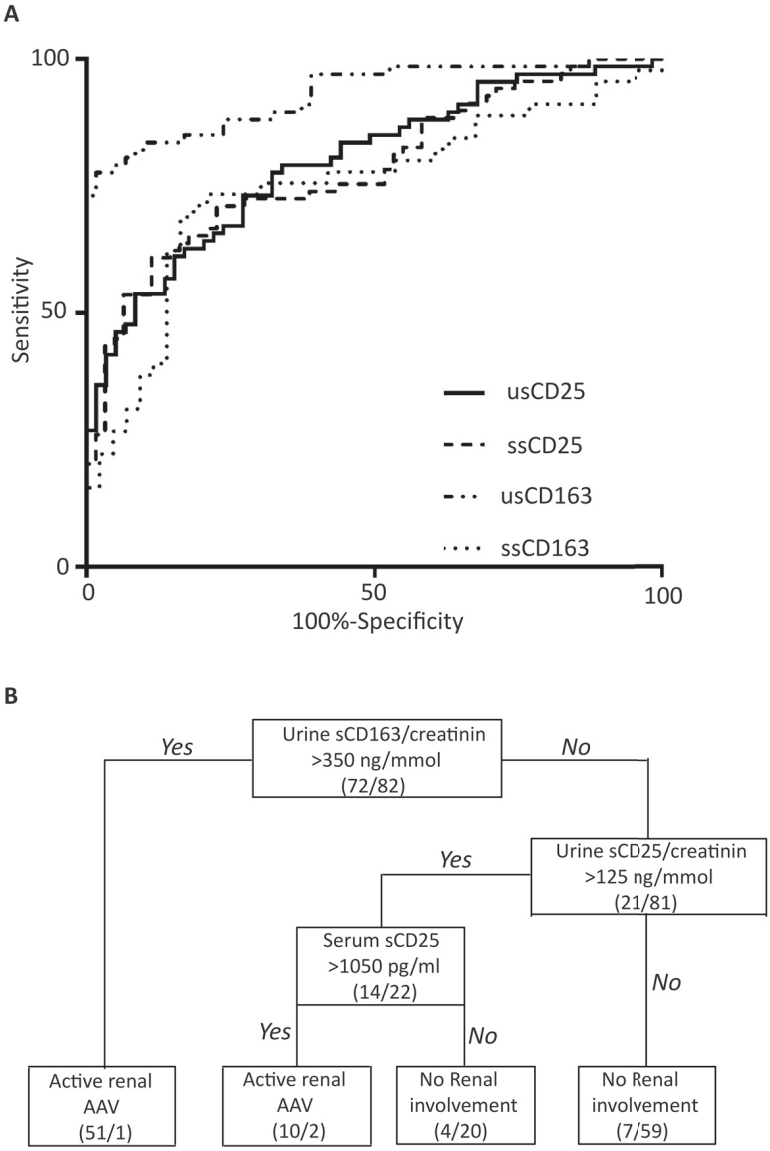

Figure 4: A combination between sCD25 and sCD163 outperforms both individual markers. Receiver operator curves (ROC) for ssCD25, usCD25 and usCD163 (A). Decision tree as generated by recursive partitioning. First number represents patients with renal active disease (n=72) and second number represents patients with non-renal active disease and remission patients (n=82) (B).

Table 3: Patient characteristics divided based on the decision tree (active patients only)

	usCD163+	ssCD25 and usCD25+ usCD163-	ssCD25 and usCD25- usCD163-
Number	51	10	11
Diagnosis/Relapse, n	36/15	6 /4	7/4
Anti-PR3-positive, n (%)	21 (40%)	8 (80%)	5 (45%)
Anti-MPO-positive, n (%)	30 (60%)	2 (20%)	6 (55%)
BVAS	15 (13 – 20)	15 (11 – 19)	14 (12 – 17)
Started induction therapy before sampling, n (%)	21 (41%)	4 (40%)	8 (73%)$^{X,\#}$
CRP (mg/l)	23 (7 – 65)	99 (59 – 190)$^{\#}$	19 (5 – 49)^{X}
Leucocytes (x10^9/l)	10.6 (8.6 – 12.9)	11.1 (8.7 - 17.0)	8.3 (6.6 – 12.0)
Serum creatinine (µmol/l)	277 (147 – 459)	90 (64 – 189)$^{\#}$	135 (108 – 158)$^{\#}$
Proteinuria (g/l)	1.5 (0.4 – 2.6)	0.3 (0.1 – 0.3)$^{\#}$	0.3 (0.1 – 0.6)

False negative patients are patients with renal active disease that tested negative for usCD163 and sCD25 (based on decision tree). $^{\#}$ significant difference compared to sCD163, ^{X} significant difference compared to sCD25.

usCD25, ssCD25, and usCD163 correlate with serum creatinine, CRP and proteinuria.

To study if ssCD25, usCD25 or usCD163 correlated to other disease markers, correlations between ssCD25, usCD25 or usCD163 and serum creatinine, serum creatinine increase and decrease, CRP, proteinuria and BVAS were tested. usCD25 correlated positively with CRP (rho=0.370, p=0.002) and negatively with serum creatinine (rho=-0.275, p=0.024) and proteinuria (rho=-0.427, p=0.005). ssCD25 correlated positively with CRP (p=0.002, rho=0.382) and serum creatinine (rho=0.240, p=0.047).

Opposite to usCD25, usCD163 levels correlated positively with serum creatinine (rho=0.546, p=0.001) and proteinuria (rho=0.430, p=0.005). No correlation with disease activity (BVAS) or ANCA titer was observed for any of the three markers. Moreover, none of the markers correlated with an increase in serum creatinine six months before or a decrease six months after active disease.

usCD25 and ssCD25 (rho=0.438, p=0.0002) as well as usCD163 and ssCD163 (rho=0.421, p=0.004) correlated positively. ssCD25 and ssCD163 also correlated positively (rho=0.421, p=0.004) but usCD25 and usCD163 did not (p=0.76).

Discussion

Early detection of renal involvement in AAV is of great clinical importance to allow early initiation of treatment to limit renal damage. Recently, usCD163 has been identified as a promising urinary marker for the detection of renal active vasculitis in the setting of a known diagnosis of AAV [7]. However, some patients with active renal vasculitis still test false negative using usCD163 alone. In the present study, we demonstrate that measuring usCD25 and ssCD25 complements usCD163 in the detection of renal active vasculitis, reducing the number of patients testing false negative. Moreover, high sCD25 levels, an indication for T cell activation and migration, might reflect an earlier disease stage characterized by high CRP levels but still limited renal damage compared to patient displaying high usCD163 levels.

There is a strong biological rationale to measure both sCD25 and sCD163 as potential markers for renal vasculitis since sCD25 reflects activation of T cells and sCD163 activation of M2 macrophages, both of which are pivotal cellular players in AAV pathogenesis. Macrophages play an important role in the development of renal damage and scarring. Moreover, macrophages are the most abundant cell type present in glomerular crescents [8, 9] and levels of usCD163 correlate to the number of macrophages present in the kidney [7].

In addition to macrophages, T cells also play a pivotal role in the induction of renal damage [26]. Here we found that usCD25 levels correlated positively to urinary T and TEM cell numbers. Not only were $CD4^+$TEM cells in the urine previously found to reflect renal involvement in AAV [17], these cells have also been suggested to play a key role in inducing kidney injury in AAV [2,26]. Depletion of $CD4^+$T cells in a mouse study significantly attenuated the development of crescentic glomerulonephritis in a model of MPO autoimmunity [26]. The observed correlation between usCD25 with both

$CD4^+$T and $CD4^+$TEM cells may reflect renal T cell accumulation in those patients. This contention is further supported by the negative correlation between circulating $CD4^+$T or $CD4^+$TEM cells and ssCD25, which suggests that after activation and CD25 shedding, T cells migrate towards the kidney.

In the patient cohorts tested here, usCD25 levels were increased in active disease, decreased upon remission, and were found to be a reasonable marker for active vasculitis (AUC 0.81). However, usCD25 alone was not specific for renal involvement, as 30% of patients without renal involvement also tested positive for usCD25. This might be due to active filtration or passive leakage of sCD25 by the kidney. However, as proteinuria was found to be negatively correlated to usCD25 passive leakage of sCD25 is unlikely.

For ssCD25, our results are in line with those from earlier studies reporting elevated ssCD25 levels during active disease that decrease upon remission [18-20]. With an AUC of 0.78, the marker potential was similar to that of usCD25, but ssCD25 was found to be more specific for renal involvement as only 10% of patients without renal disease tested positive. Previously, O'Reilly et al showed that usCD163 alone is a potent marker for renal active disease, and with a sensitivity between 73-96% it outperforms ssCD25 and usCD25 [7]. In contrast to O´Reilly we found, in our validation cohort, significant higher ssCD163 levels in patients with active renal disease compared to patients in remission. These results however could not be validated in a larger validation cohort and could be partly explained by differences in the number of patients receiving immunosuppressive treatment at the time of sampling.

Interestingly, in our cohort, ten patients with active renal vasculitis who tested negative for usCD163 had high ssCD25 and usCD25 levels. Clinical parameters differed between these two groups. Patients negative for usCD163 but with high usCD25 and ssCD25 levels had higher CRP levels and lower serum creatinine and proteinuria. These results suggest that levels of sCD25 may increase at an earlier stage of the disease process when renal injury is still limited. In contrast, usCD163 correlated positively with proteinuria and serum creatinine, which suggests that usCD163, might be associated with more established renal damage. These results indicate that usCD25, ssCD25, and

usCD163 have different kinetics during the development of renal injury in AAV that may reflect different stages of the inflammatory process. This is emphasized by the lack of correlation between usCD25 and usCD163.

Based on our results, measuring usCD25 and ssCD25 in combination with usCD163 could improve the detection of renal flares in AAV patients before extensive renal damage has occurred. However, fifteen percent of the patients with renal active disease still tested false negative when this combination was used. This observation can be partly explained by the higher number of patients that received induction therapy before sampling in the false negative group compared to the true positive group (73% vs 41%). In patients that received induction therapy days before sampling levels of ssCD25 were significantly lower and usCD25 levels tended to be lower, which might explain these false negative results.

A limitation of using usCD25 and ssCD25 as well as usCD163 as indicators of disease activity is that these markers are not disease specific. Previous studies have demonstrated elevated usCD25 and ssCD25 levels in septic patients with or without renal involvement [27-29], and increased usCD25 and usCD163 levels have been reported in lupus nephritis [21,30]. Although these markers are thus not specific for renal active vasculitis in AAV, and therefore cannot be used to diagnose AAV, their utility as markers of active renal vasculitis in patients with an established diagnosis of AAV is promising. An additional limitation of our study is its retrospective design. Therefore, additional prospective studies are necessary to substantiate the value of combined measurement of sCD163 and sCD25 in the detection of renal active AAV.

In conclusion, our results indicate that usCD25 and ssCD25 complements usCD163 in the detection of renal active vasculitis in AAV patients. Our results also suggest that elevated usCD25 and ssCD25 levels reflect an earlier stage of development of renal vasculitis in AAV patients which could be of clinical importance as an early sign of active disease. Further studies should be carried out to confirm and extend our findings in a prospective manner.

Disclosure

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Supplemental table 1: Effect of induction or maintenance treatment

	Active AAV		
	Induction therapy	**Maintenance treatment**	**Untreated**
usCD25	59 (40-120) *	126 (77-340)	189 (92-455)
ssCD25	798 (403-1366)*	1057 (185-1595)	1369 (738-2149)
usCD163	464 (248-798)	481 (300-885)	575 (280-2341)
ssCD163	255 (177-432)*	574 (295-806)	679 (633-1039)

	Active AAV without renal involvement		
	Induction therapy	**Maintenance treatment**	**Untreated**
usCD25	125 (37-140)	57 (30-97)	131 (79-154)
ssCD25	334 (133-481)	428 (250-643)	393 (352-861)
usCD163	114 (40-148)	61 (23-158)	104 (57-185)
ssCD163	539 (456-621)	328 (239-419)	332 (189-447)

	Active AAV without renal involvement		
	Induction therapy	**Maintenance treatment**	**Untreated**
usCD25	-	135 (55-160)	85 (40-152)
ssCD25	-	483 (255-791)	524 (290-680)
usCD163	-	70 (61-115)	49 (20-102)
ssCD163	-	580 (204-881)	463 (293-810)

* significant difference compared to untreated group.

Supplemental table 2: Statistical analysis of traditional markers for renal active AAV

Marker	AUC	Sensitivity	Specificity	Cut-off value
Proteinuria	0.73	53.6	82.7	>0.5 g/l
Hematuria#	0.77	85.7	75.0	>10 RBC/ µl
Serum creatinine	0.76	68.1	72.5	>140 µmol/l
CRP	0.79	77.9	71.6	> 6.5 mg/l
CRP/ Serum Creatinine	-	86.1	51.2	
CRP/Proteinuria	-	82.0	65.9	

Only data from the Groningen cohort was available for this analysis